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Maintaining the Line of Defense: Regeneration of Cuvierian Tubules in the Sea Cucumber *Holothuria forskali* (Echinodermata, Holothuroidea)

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Abstract. When irritated, individuals of the sea cucumber *Holothuria forskali* expel a few Cuvierian tubules which lengthen, instantly become sticky, and rapidly immobilize most organisms with which they come into contact. After expulsion, the lost tubules are readily regenerated. When only a few tubules have been expelled, there is often a latent period before the regeneration starts. In contrast, when many tubules have been expelled, the regenerative process starts immediately but proceeds in successive waves of 10 to 30 tubules that begin to regenerate at 10-day intervals. However, in all cases, the complete regeneration of a given tubule takes about 5 weeks and may be divided into three successive phases: an initial repair phase including the overall 48-h post-autotomy period, a true regenerative phase taking about 4 weeks to complete, and a growth phase of about one more week. Initial regeneration events occur by epimorphosis, cell proliferation being essential to the regenerative process, whereas late events occur mainly by morphallaxis, with migration of the newly differentiated cells. The mesothelium is the tissue layer in which cell proliferation is the most precocious and the most important, involving both peritoneocytes and undifferentiated cells (which seem to be dedifferentiated peritoneocytes). As regeneration proceeds, the percentage of undifferentiated cells regularly decreases in parallel with the differentiation of granular (adhesive-secreting) cells and myocytes. The myocytes then separate off from the mesothelium and migrate within the connective tissue layer. Three types of pseudopodial cells follow one another in the tubule connective tissue during regeneration. Type 1 cells have all the characteristics of echinoderm phagocytes and may have a fibroclastic function, cleaning the connective tissue compartment before new collagen synthesis starts. Type 2 cells are rather undifferentiated and divide actively. The presence of type 3 cells is closely associated with the appearance of collagen fibers, and it is suggested that they have a fibroblastic function. In the inner epithelium, cells also divide actively, but only those in which spherules have not yet differentiated in the basal intraconnective processes. It appears, therefore, that in the three tissue layers of the tubules, regeneration proceeds by cell dedifferentiation, then proliferation, and finally by differentiation. Cuvierian tubules thus constitute a very efficient defensive mechanism: their large number, sparing use, and particular regeneration dynamics make them an almost inexhaustible line of defense maintained at limited energy cost.

Introduction

Several species of holothuroids (sea cucumbers), all belonging to the order Aspidochirotida, possess a very specialized defensive system: the so-called Cuvierian tubules (Flammang, 1996). This system consists of several hundred tubules whose proximal ends attach to the basal part of the left respiratory tree and whose distal, blind ends float freely in the coelomic cavity. When irritated, the animal curves its aboral end toward the irritating object and undergoes a general contraction. The anus opens, the wall of the cloaca tears, and the free ends of the Cuvierian tubules, together with coelomic fluid, are expelled through the tear and the

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anus. The emitted tubules lengthen, instantly become sticky, and rapidly immobilize most organisms with which they come into contact (VandenSpiegel and Jangoux, 1987). Finally, the expelled tubules autotomize at their attachment point on the left respiratory tree and are left behind as the holothuroid crawls away (Müller *et al.*, 1970; VandenSpiegel and Jangoux, 1987). As only a portion of the tubules are emitted at one time, the total number may suffice for several responses. After expulsion, the lost tubules are readily regenerated (Hyman, 1955).

The fine structure of the Cuvierian tubules in the species *Holothuria forskali* has been described by VandenSpiegel and Jangoux (1987). Quiescent Cuvierian tubules consist of an outer mesothelium, an inner epithelium, and between them, a thick connective tissue layer that includes muscle fibers. The mesothelium is a pseudostratified epithelium made up of two cell layers, namely an apical layer of ciliated peritoneocytes and a basal layer of granular cells filled with densely packed lipoproteic granules. Together, these layers form conspicuous transverse folds that penetrate the underlying connective tissue. Circular and longitudinal myocytes occur under the tips of the mesothelial folds. These cells divide the connective tissue into a thin outer layer (where collagen fibers are directed perpendicularly to the tubule long axis) and a thick inner layer (where collagen fibers are arranged in helixes parallel to the tubular long axis). Nerve processes are associated with the longitudinal muscles; they also occur between the spirally arranged collagen fibers. The tubule lumen is narrow with a highly convoluted limiting epithelium. Epithelial cells are not ciliated; they have basal processes containing enlarged spherules whose contents include mucosubstances and proteins.

The histological organization of Cuvierian tubules, which is quite different from that of the left respiratory tree that bears them, appears to be functionally important for their correct operation (VandenSpiegel and Jangoux, 1987). However, as pointed out by Smiley (1994), the histogenesis of these intriguing organs is virtually unknown. Similarly, nothing is known about the renewal of the Cuvierian tubule stock after expulsion and, consequently, on the way holothuroids maintain their line of defense. We have thus investigated the process of Cuvierian tubule regeneration in the holothuroid *Holothuria forskali*. Our aims were (i) to study the dynamics of the regenerative process and to describe macroscopically the different regeneration stages; (ii) to detail the installation of the different tissue layers, using electron microscopy; and (iii) to determine, through immunohistochemistry and autoradiography, the origin of the cells involved in tubule regeneration.

Materials and Methods

Individuals of *Holothuria forskali* (Delle Chiaje, 1823) were collected at a depth of 20 to 30 m by scuba diving in the bay of Morlaix (Brittany, France). They were transported to the Marine Biology Laboratory of the University of Mons-Hainaut, where they were kept in a marine aquarium with closed circulation (13°C, 33‰ salinity).

Induction of tubule expulsion and sampling of regenerating tubules

The expulsion of Cuvierian tubules was induced mechanically by pinching the dorsal integument of the holothuroids with forceps. Two sets of regenerating animals were investigated: the first set comprised 15 individuals that had been stimulated only once and had expelled about 15 tubules (gentle stimulation); the second set consisted of 25 individuals that had been stimulated repeatedly to induce the expulsion of about 300 tubules (strong stimulation). After tubule expulsion, all the animals were returned to the aquarium and dissected at various times to provide samples for observation macroscopically and with transmission electron microscopy (TEM). Tubules in the process of regeneration were dissected from each set of cucumbers every day for 6 days, and then weekly for 8 weeks. Before dissection, the animals were anesthetized for 1 h in 0.2% propylene phenoxytol (Nipa laboratories, UK; see Hill and Reinschmidt, 1976) in seawater. For the immunohistochemical and autoradiographical studies of Cuvierian tubule regeneration, 10 of the strongly stimulated sea cucumbers were put aside in a separate aquarium for 1 month.

Light and transmission electron microscopy

Regenerating tubules were fixed for 3 h at 4°C in 3% glutaraldehyde in cacodylate buffer (0.1 *M*, pH 7.8; adjusted to 1030 mOsm with sodium chloride), then rinsed in cacodylate buffer and post-fixed for 1 h in 1% osmium tetroxide in the same buffer. After a final wash in buffer, the podia were dehydrated in graded ethanol and embedded in Spurr. Semithin sections (1 μm in thickness) were cut on a Reichert Om U2 ultramicrotome equipped with a glass knife and then stained according to the method of Humphrey and Pittman (1974). Ultrathin sections (40–70 nm) were cut with a Leica UCT ultramicrotome equipped with a diamond knife, stained with uranyl acetate and lead citrate, and observed with a Zeiss EM 10 transmission electron microscope.

Immunohistochemistry

The BrdU/anti-BrdU method was used to study DNA synthesis in the regenerating tubules. After one month of regeneration, five of the strongly stimulated holothuroids were injected intracoelomically with 2 ml of a 2% solution of BrdU (5-bromo-2-deoxyuridine; Aldrich Chemie) in filtered seawater (0.45 μm; Millipore). One hour after injection, the individuals were dissected and their regenerating

tubules fixed for 6 h in Allen-Bouin's fluid (Gabe, 1968). They were then rinsed thoroughly in running tap water, dehydrated in graded ethanol, embedded in Paraplast, and sectioned (5 μm). BrdU-containing DNA in the nuclei of proliferating cells was detected by the immunogold-silver staining (IGSS) method (Hacker *et al.*, 1985; adapted for echinoderm tissues in VandenSpiegel *et al.*, 1991). The sections were treated with trypsin (0.1% trypsin in 0.01 *M* sodium barbital, 0.15 *M* NaCl, pH 8.0) for 50 min at 37°C, followed by acid hydrolysis in 3 *M* HCl at 60°C for 30 min. This treatment denatures the DNA and unmasks hidden epitopes. After rinsing in distilled water, the sections were preincubated for 20 min in 5% normal goat serum in 0.01 *M* sodium barbital, 0.15 *M* NaCl, pH 7.4 (veronal buffer-NGS), then incubated overnight at 4°C with mouse anti-BrdU monoclonal antibodies (DAKO A/S) diluted 1:30 in veronal buffer-NGS. After several washes in phosphate-buffered saline (PBS), the sections were incubated for 1 h at room temperature in goat anti-mouse immunoglobulins conjugated to 5 nm gold particles (Auroprobe LM; Amersham) diluted 1:40 in PBS-NGS. Gold particles bound to the immunocomplexes were visualized by a silver precipitation reaction, using a commercial kit (Intense SE TMII; Amersham). The reaction was stopped with distilled water and the sections were counterstained with hemalun and eosin.

Autoradiography

Cell proliferation was also monitored using autoradiography. Tritiated thymidine (150 μCi of a 1 mCi/ml aqueous solution of 5′-^{3}HT; Amersham) was mixed with 1 ml of filtered seawater and injected, through the holothuroid integument, into the coelomic cavity. This injection was performed on five strongly stimulated individuals that had started regenerating Cuvierian tubules one month earlier. One hour after injection, the holothuroids were dissected, and the regenerating tubules were fixed and embedded as for TEM (see above). Semithin sections (1-μm thick) were collected on glass slides and stored in distilled water. All subsequent manipulations took place in a darkroom. The slides were dipped in photographic emulsion (Nuclear research emulsion L5; Ilford) diluted 1:1 in distilled water at 45°C. After air-drying, the coated slides were stored in light-proof boxes at 4°C for 10 days. Exposed slides were developed for 4 min at room temperature in Kodak D19 diluted 1:2 in distilled water, transferred to a stop-bath (2% acetic acid) for 30 s, followed by fixation (10% Acidofix) for 5 min. After rinsing in running tap water, the autoradiographed sections were stained with toluidine blue.

Counting and identification of proliferating cells

Labeled nuclei were counted in sections of regenerating tubules that were processed either by immunohistochemistry or by autoradiography. For each regeneration stage, a labeling index (LI) was determined in the three main tissue layers of the tubules (*i.e.*, the mesothelium, the connective tissue layer, and the inner epithelium). This index represents the percentage of labeled nuclei (LN) compared to the total number of counted nuclei (TN); it is calculated according to the formula: $LI = (LN/TN) \times 100$. For each stage, four counts, each comprising a maximum of 100 nuclei, were done on sections coming from four different regenerating tubules from a single holothuroid.

To identify proliferating cells we used the procedure of Laurent *et al.* (1988), which relies on the fact that tissues labeled with ^{3}HT and embedded in Spurr resin can be observed by both light and electron microscopy. For each regeneration stage, we used three to four ultrathin sections directly followed by one semithin section. The former were observed in TEM, whereas the latter was processed for autoradiography (see above). In most cases, the shape of the labeled cells, as it appeared on the semithin section, was characteristic enough to allow their localization on the ultrathin section. It was therefore possible to identify the cell types involved in DNA synthesis.

Results

Macroscopic observations

Regeneration of the Cuvierian tubules in *Holothuria forskali* was observed after two types of stimulations: gentle stimulations inducing the expulsion of about 15 tubules and strong stimulations inducing the expulsion of about 300 tubules. After a gentle stimulation, tubule expulsion is not always followed by immediate regeneration of new tubules; rather, a latent period of up to 20 days was observed. In addition, the beginning of the regeneration could not be detected without sacrificing the animal. Therefore, a regeneration that followed a gentle stimulation was difficult to study. However, data collected from the staggered dissections have allowed us to estimate that the duration of the regeneration is at least 4 weeks, and that all of the autotomized tubules regenerate synchronously. After a strong stimulation, on the other hand, regeneration starts immediately, although only for a limited number of tubules. Indeed, the regeneration proceeds by groups of 10 to 30 tubules; the beginning of the regeneration in one group being separated from that of the following one by 5 to 10 days. Therefore, about one month after the induction of a massive tubule autotomy, holothuroids have tubules at several stages of regeneration (Fig. 1).

The observation of Cuvierian tubule regeneration after a strong stimulation has allowed us to describe five successive stages in the expulsion-autotomy-regeneration cycle (Fig. 2):

Stage 1 corresponds to the healing of the damaged tissues followed by the appearance, 2 to 6 days after autotomy, of

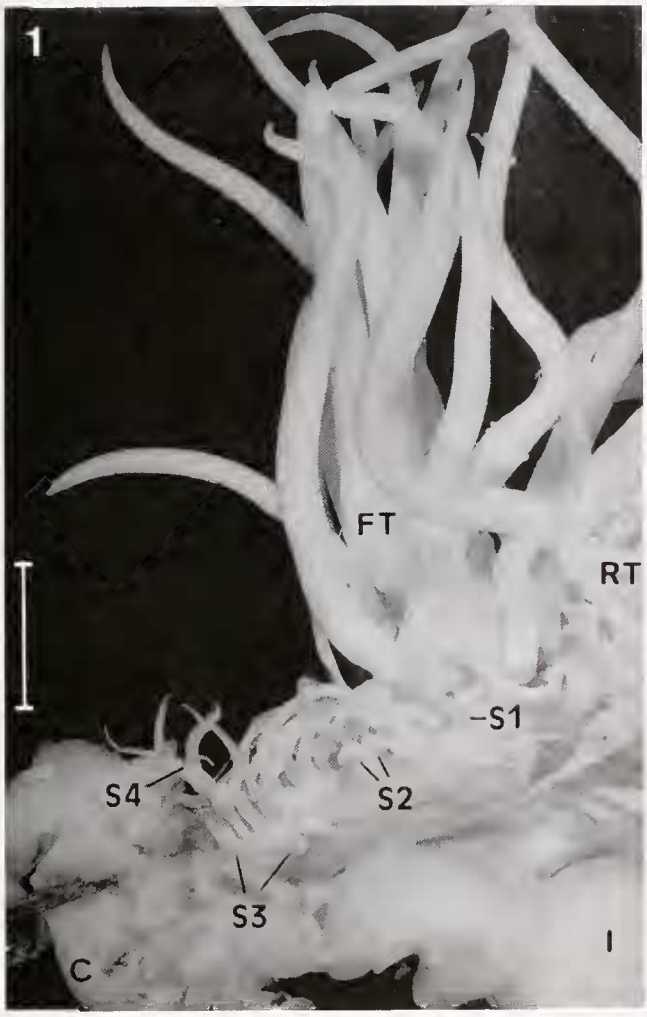


Figure 1. General aspect of regenerating Cuvierian tubules in *Holothuria forskali* 3 weeks after a massive tubule expulsion, showing tubules at different stages of regeneration (bar = 0.5 cm). C, cloaca; FT, fully developed tubule; I, intestine; RT, left respiratory tree; S1–S4, stages 1–4.

a thickening of the wall of the basal part of the left respiratory tree, at the place where the expelled tubules were attached (S1; Figs. 1 and 2).

Stage 2 corresponds to the formation, 8 to 10 days after autotomy, of a translucent spherical structure measuring from 0.1 to 0.4 mm in diameter (S2; Figs. 1 and 2).

Stage 3 corresponds to the development, 10 to 14 days after autotomy, of a translucent club-shaped structure ranging from 1 to 3 mm in length for a maximum diameter of 0.3 mm (S3; Figs. 1 and 2).

Stage 4 corresponds to the transformation of the regenerating structure, 14 to 21 days after autotomy, into a small pale-pink tubule measuring from 4 to 12 mm in length (S4; Figs. 1 and 2). From this stage on, the shape of the regenerating tubule remains constant, only the length and diameter of the tubule increase.

Stage 5 corresponds to the differentiation, 21 to 28 days after autotomy, of a functional tubule that possesses a short basal peduncle and measures from 14 to 22 mm long for a diameter of about 0.65 mm (S5; Fig. 2). Although they are only half the size of a fully developed tubule, regenerating tubules at stage 5 are able to lengthen and become sticky. They reach their full size 28 to 35 days after autotomy.

Ultrastructure of regenerating tubules

Immediately after their expulsion and lengthening, Cuvierian tubules are autotomized at the narrow peduncle that attaches them to the left respiratory tree. The resulting wound is relatively limited and is rapidly closed by migration of mesothelial cells on the coelom side and epithelial cells on the side of the respiratory tree lumen. At the same time, the connective tissue is invaded by very active phagocytes that remove cell debris from the tissue. These large cells have many cytoplasmic inclusions, such as phagosomes, secondary lysosomes, and residual bodies.

Stage 1. Regeneration *sensu stricto* takes place right after wound healing and starts with the formation of a small mesothelial bud that appears as a local thickening of the wall of the basal part of the left respiratory tree. This bud consists of a mass of cells forming a pseudostratified mesothelium in which an outer layer of ciliated adluminal cells (peritoneocytes) covers a few layers of undifferentiated cells (Fig. 3). Peritoneocytes are tack-shaped cells whose apical part lines the coelomic cavity. They are bound to-

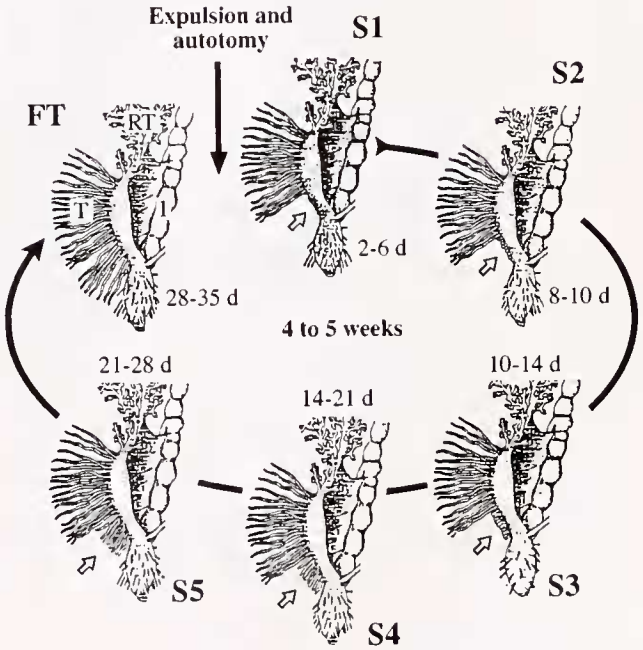
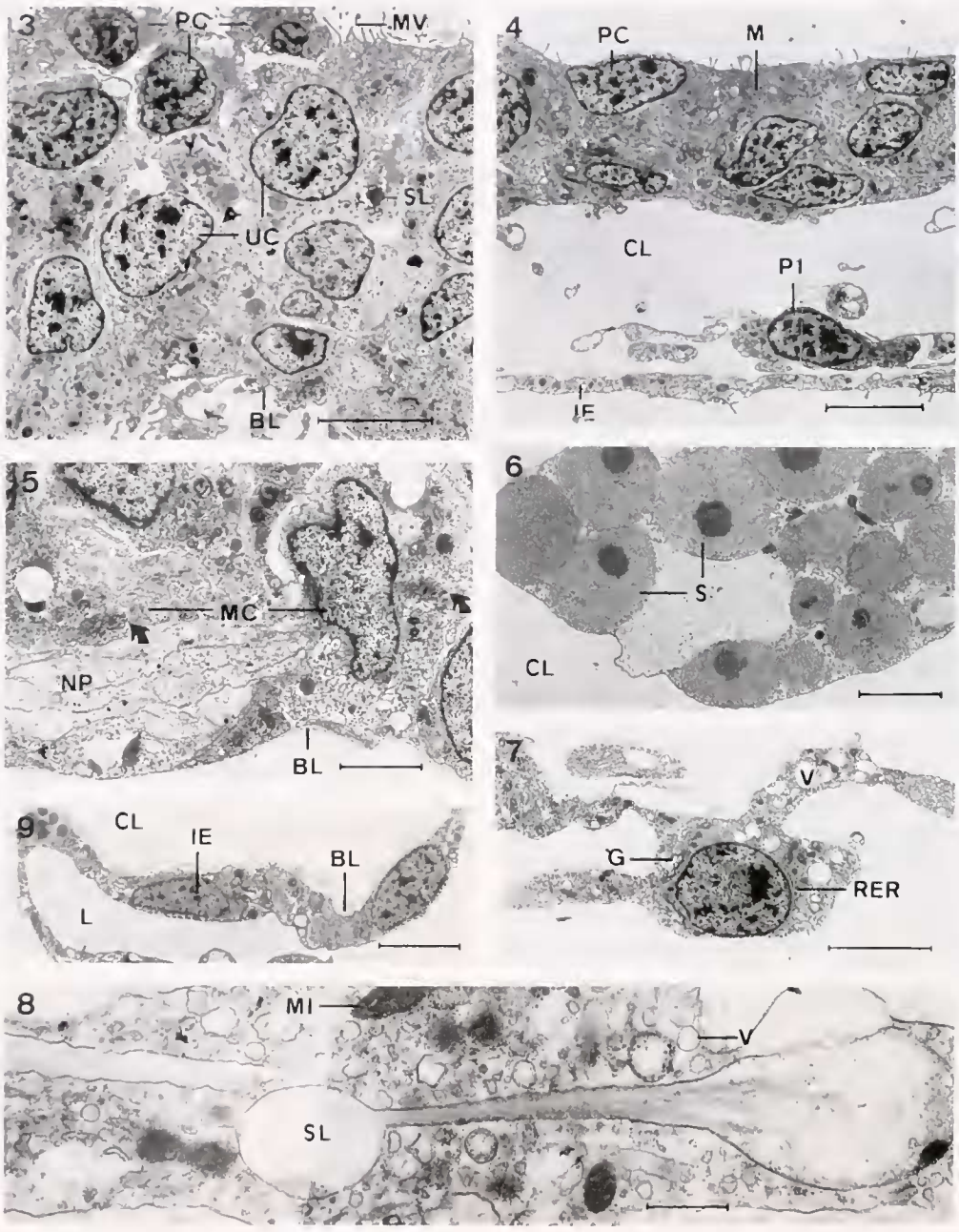


Figure 2. Chronology of the regeneration stages of Cuvierian tubules in *Holothuria forskali*. Open arrows point to the regenerating tubules. FT, fully-developed tubules; I, intestine; RT, left respiratory tree; T, Cuvierian tubules; S1–S5, stages 1–5.



Figures 3–9. *Holothuria forskali.* Ultrastructure of stage 1 and 2 regenerating Cuvierian tubules. BL, basal lamina; CL, connective tissue layer; G, Golgi apparatus; IE, internal epithelium; L, lumen; M, mesothelium; MC, myocyte; MI, mitochondrion; MV, microvillus; NP, nerve plexus; P1, type 1 pseudopodial cell; PC, peritoneocyte; RER, rough endoplasmic reticulum; S, spherule; SL, secondary lysosome; UC, undifferentiated cell; V, vesicle. **Figure 3.** Mesothelium at stage 1 (bar = 5 μm). **Figure 4.** Longitudinal section through the wall of a stage 2 regenerating tubule (bar = 5 μm). **Figure 5.** Detailed view of the basal part of the mesothelium showing the myocytes and the nerve plexus (bar = 2 μm). Arrows indicate bundles of myofilaments. **Figure 6.** Intraconnective spherulocyte (stage 2; bar = 2 μm). **Figure 7.** Intraconnective type 1 pseudopodial cell (stage 2; bar = 4 μm). **Figure 8.** Detailed view of the cytoplasm of a type 1 pseudopodial cell (stage 2; bar = 0.5 μm). **Figure 9.** Longitudinal section through the inner epithelium (stage 2; bar = 4 μm).

gether by an apical zonula adhaerens. Each peritoneocyte bears a single cilium (about 10 μm long) and a few scattered microvilli (Fig. 3). The apical part of the cell houses the nucleus, a juxtanuclear Golgi complex, a well-developed rough endoplasmic reticulum (RER), numerous free ribosomes, some lipid granules, and many mitochondria. Large

secondary lysosomes (1 to 1.5 μm in diameter) also occur in the apical cytoplasm. The basal part of the cell, which is thin and elongated, traverses the undifferentiated cell layers before contacting the basal lamina through a hemidesmosome-like structure. Undifferentiated cells are cube-shaped and have a high nuclear-cytoplasmic ratio (Fig. 3). However, although reduced, the perinuclear cytoplasm encloses a well-developed Golgi apparatus and RER, many mitochondria and free ribosomes, lipid granules, and numerous secondary lysosomes (Fig. 3). Undifferentiated cells are connected to the basal lamina through hemidesmosome-like structures, but they are neither bound together nor bound to the peritoneocytes.

Stage 2. At the end of this stage, the regenerating structure already has the three tissue layers characteristic of a non-regenerating Cuvierian tubule, *i.e.*, a mesothelium, a connective tissue layer, and an inner epithelium (Figs. 4, 10).

The general organization of the mesothelium is similar to the one observed in stage 1 except that some myocytes occur among the undifferentiated cells (Figs. 5, 10). Myocytes are cube-shaped. They have a large nucleus and enclose one bundle of myofilaments in their cytoplasm (Fig. 5), which also contains a well-developed RER and numerous mitochondria. No junction connecting the myocytes to the other mesothelial cells was observed. However, each myocyte is anchored to the basal lamina by a hemidesmosome-like structure. A discrete nerve plexus is associated with the mesothelium of stage 2 regenerating tubules. It consists of nerve processes, preferentially located close to the myocytes (Figs. 5, 10).

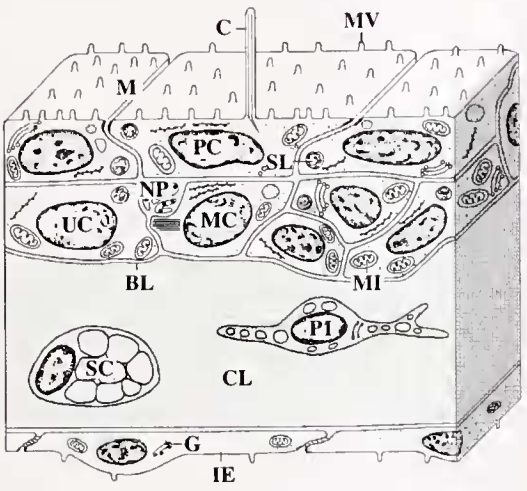


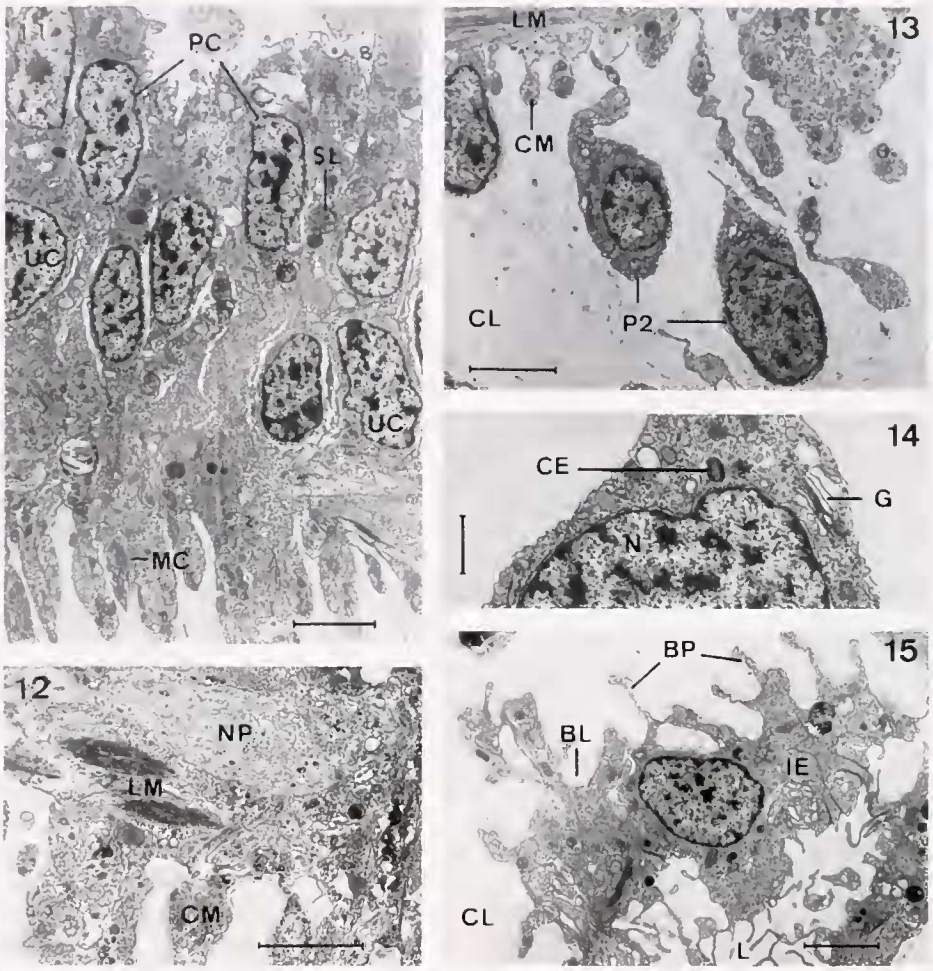
Figure 10. *Holothuria forskali.* Reconstruction of a longitudinal section through the wall of a stage 2 regenerating Cuvierian tubule (not to scale). BL, basal lamina; C, cilium; CL, connective tissue layer; IE, internal epithelium; G, Golgi apparatus; M, mesothelium; MC, myocyte; MI, mitochondrion; MV, microvillus; NP, nerve plexus; PI, type 1 pseudopodial cell; PC, peritoneocyte; SC, spherulocyte, SL, secondary lysosome; UC, undifferentiated cell.

Encompassed between the basal lamina of the mesothelium and that of the inner epithelium, the thin connective tissue layer (about 7.5 μm in thickness) is poor in collagen fibers, but contains many mesenchymatous cells, both spherulocytes and type 1 pseudopodial cells (Figs. 6, 7, 10). Spherulocytes are ovoid to spherical cells, 5 to 10 μm in diameter. Their cytoplasm is packed with spherules that average 3 μm in diameter and enclose an electron-lucent material (Fig. 6). Type 1 pseudopodial cells possess a central, nucleus-containing cell body from which two to three thin cell processes radiate (Figs. 4, 7). Their cytoplasm encloses one or two juxtanuclear Golgi stacks, a well-developed RER, a few lysosomal bodies, many mitochondria, and numerous clear vesicles of various sizes. In some micrographs, large secondary lysosomes enclosing partly decomposed collagen fibrils were observed (Fig. 8).

The inner epithelium delimits a quite large lumen. It consists of only one cell type that strongly resembles the epithelial cells of the respiratory tree at the level where Cuvierian tubules are inserted. These cells are non-ciliated cells that bear a few apical microvilli (Figs. 4, 9, 10). They are connected together through junctional complexes consisting of an apical zonula adherens and a subapical septate desmosome, but they do not attach to the basal lamina. Epithelial cells are flat (2.4 μm in the nucleus-containing thickest part). Their cytoplasm contains a Golgi apparatus, a few scattered mitochondria, and some lipid granules.

Stage 3. The mesothelium is pseudostratified and still comprises peritoneocytes, undifferentiated cells, and myocytes (Figs. 11, 12, 16). Peritoneocytes and undifferentiated cells are similar to those observed in stages 1 and 2. In contrast, myocytes have clearly increased in number and can be divided into two sets according to the orientation of their bundle of myofilaments, either circular or longitudinal. Circular myocytes are always located beneath longitudinal myocytes. They possess a basal cytoplasmic process that penetrates the underlying connective tissue layer and encloses the bundle of myofilaments (Figs. 12, 13). The nerve plexus is more developed than in the previous stage and still preferentially associated with the myocytes (Figs. 12, 16).

The connective tissue layer has increased in thickness (to about 30 μm) and, at this stage, makes up three-quarters of the total thickness of the regenerating tubule wall. As in the previous stage, the connective tissue layer is poor in collagen fibers and contains numerous mesenchymatous cells. Spherulocytes, however, are no longer present; only pseudopodial cells remain. These cells are of two types: type 1 cells, identical to those observed at the previous stage, and type 2 cells; the latter being, by far, the more abundant. Type 2 pseudopodial cells have a particular distribution; they are mainly located just below the basal lamina of the mesothelium (Figs. 13, 16). They have a teardrop shape, the tapered part forming a single process that always faces the basal lamina. These cell processes were sometimes ob-



Figures 11–15. *Holothuria forskali.* Ultrastructure of stage 3 regenerating Cuvierian tubules. BL, basal lamina; BP, epithelial cell basal process; CE, centriole; CL, connective tissue layer; CM, circular myocyte; IE, internal epithelium; G, Golgi apparatus; L, lumen; LM, longitudinal myocyte; MC, myocyte; N, nucleus; NP, nerve plexus; P2, type 2 pseudopodial cell; PC, peritoneocyte; SL, secondary lysosome; UC, undifferentiated cell. **Figure 11.** Longitudinal section through the mesothelium (bar = 3 μm). **Figure 12.** Detailed view of the myocytes (bar = 3 μm). **Figure 13.** Intraconnective type 2 pseudopodial cells (bar = 3 μm). **Figure 14.** Detailed view of a type 2 pseudopodial cell (bar = 0.5 μm). **Figure 15.** Transverse section through the inner epithelium (bar = 3 μm).

served contacting the intraconnective basal processes of the circular myocytes (Fig. 13). Type 2 pseudopodial cells have a high nuclear-cytoplasmic ratio. Their reduced perinuclear cytoplasm encloses a Golgi apparatus as well as a pair of centrioles (Fig. 14).

The inner epithelium is only slightly different from that seen in stage 2. The epithelial cells have a more irregular shape and develop two to three basal processes penetrating the underlying connective tissue layer (Figs. 15, 16). These processes are lined by the basal lamina but, as previously, there is no attachment structure between the epithelial cells and this basal lamina.

Stage 4. At this stage of regeneration, the mesothelium develops transversal folds that penetrate the connective tissue layer (Figs. 17, 20). Three cell types may be recognized in the mesothelium: peritoneocytes, granular cells, and myocytes. The undifferentiated cells are no longer present. Peritoneocytes form the coelomic lining and always cover the granular cells. They are tack-shaped with a flattened apical part and a thin elongated basal part. They are now attached to each other through junctional complexes consisting of an apical zonula adherens and a subapical septate desmosome, and to the basal lamina through hemidesmosome-like structures. The nucleus is located in the apical part of the cell where the cytoplasm contains a Golgi apparatus and a few mitochondria (Fig. 17). Granular cells are very flattened and form a subapical cell layer that is accordion-pleated perpendicularly to the long axis of the tubule, each pleat penetrating the underlying connective tissue. The cytoplasm of these cells contains many electron-dense gran-

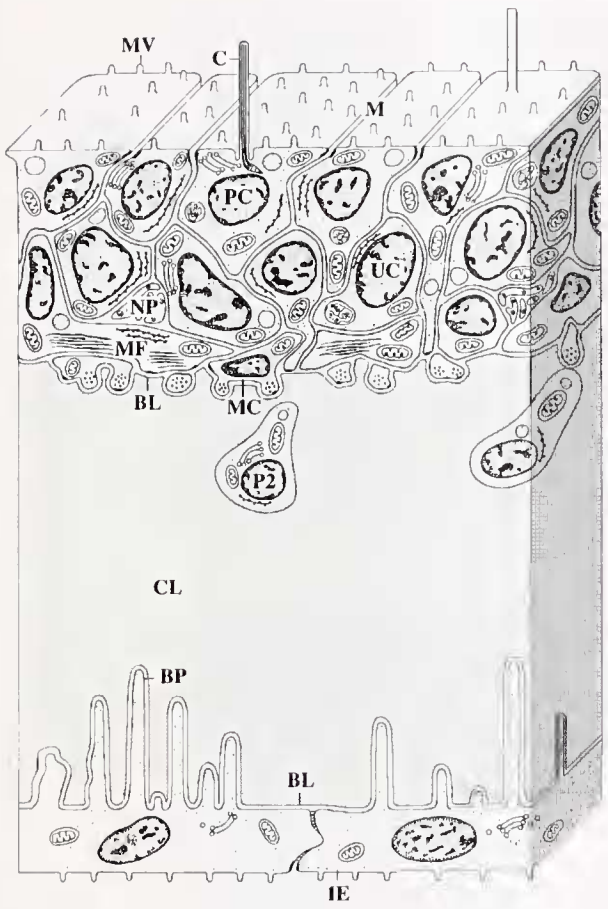


Figure 16. *Holothuria forskali.* Reconstruction of a longitudinal section through the wall of a stage 3 regenerating Cuvierian tubule (not to scale). BL, basal lamina; BP, epithelial cell basal process; C, cilium; CL, connective tissue layer; IE, internal epithelium; M, mesothelium; MC, myocyte; MF, bundle of myofilaments; MV, microvillus; NP, nerve plexus; P2, type 2 pseudopodial cell; PC, peritoneocyte; UC, undifferentiated cell.

ules that range from 0.1 to 0.3 μm in diameter and often occur in the vicinity of a large Golgi apparatus and of a well-developed RER (Fig. 17). Many of the cisternae of the RER are distended and filled with an amorphous material. The nucleus is egg-shaped and usually located in the lowermost part of the cell (*i.e.*, at the base of the pleats). No cellular junctions were observed between the granular cells or between the granular cells and peritoneocytes. However, the granular cells attach to the basal lamina through a hemidesmosome-like structure. Myocytes are located either in the most basal part of the mesothelium (*i.e.*, at the base of the folds), or here and there within the connective tissue layer, where they form an intraconnective muscle layer (Figs. 17, 20). The circular myocytes are always basal to the longitudinal ones. A single basal lamina lines both types of myocytes as well as the rest of the mesothelium. A well-developed nerve plexus is present and is mostly associated with the longitudinal myocytes, whether they are intramesothelial or within the connective tissue (Figs. 17, 20).

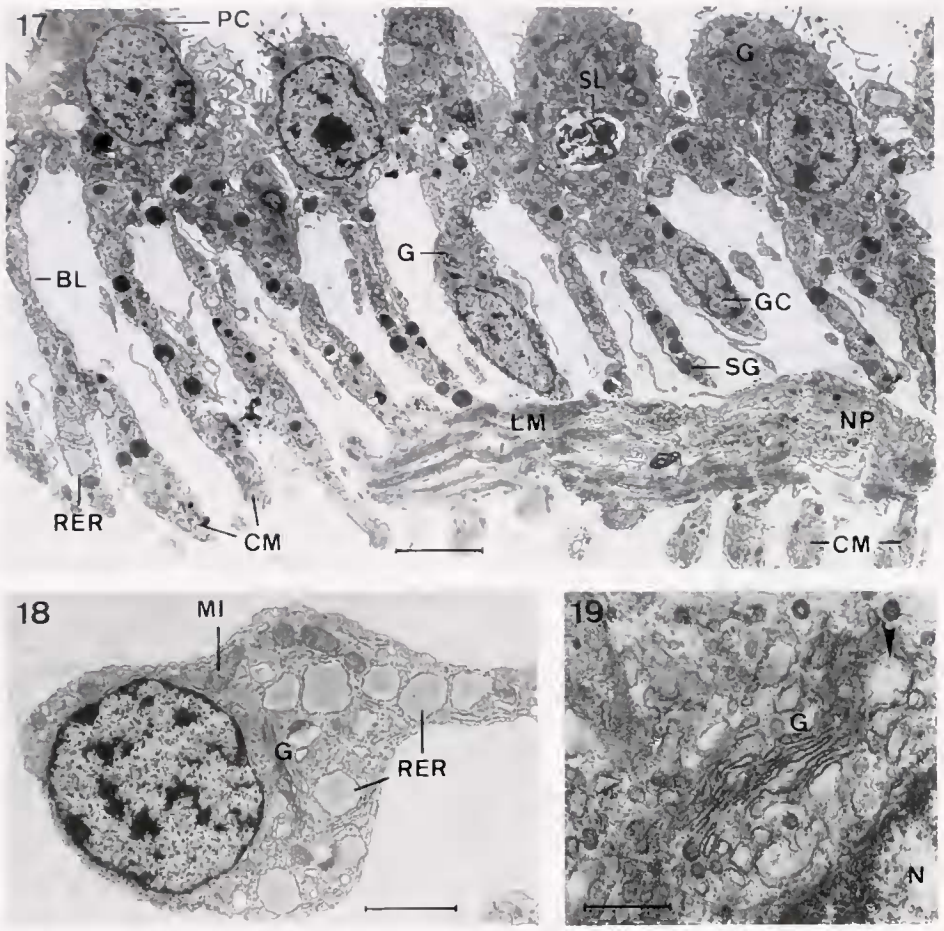
The thick connective tissue layer is, from place to place, divided into an outer area and an inner area by the presence of the intraconnective muscle layer. The outer area is thin (about 12 μm thick) and encloses very few collagen fibers and cells. The inner area, on the other hand, is much thicker (about 120 μm thick); it contains many collagen fibers, which show no tridimensional organization, and numerous pseudopodial cells (Fig. 20). Most of the latter are type 3 cells, although a few type 2 cells may still be observed. Type 3 pseudopodial cells possess a central nucleus-containing cell body from which one to three cell processes radiate (Fig. 18). Their characteristic feature is an extensive RER with some very distended cisternae filled with an electron-lucent amorphous material (Fig. 18). Their cytoplasm also contains numerous mitochondria and a well-developed Golgi apparatus. On some micrographs, the Golgi stacks show cisternae ending with spheroidal distentions that enclose a fuzzy material of low electron density (Fig. 19).

The inner epithelium is similar to the one described in stage 3 (Fig. 20).

Stage 5. At this stage, the ultrastructure of the regenerating tubule is very similar to that of a non-regenerating quiescent Cuvierian tubule (or fully developed tubule); the tubules in these two stages differ mostly in their size.

The mesothelium consists of peritoneocytes that cover elongated granular cells. Together they form the conspicuous transversal folds that are characteristic of the nonregenerating tubule mesothelium (Figs. 21, 25). The muscle layer is now completely separated from the mesothelium and is located in the outer part of the connective layer, just under the tips of the mesothelial folds (Figs. 21, 25). This layer comprises outer longitudinal and inner circular myocytes, and it divides the connective tissue layer into a thin outer area and a thick inner area. Numerous nerve processes are associated with the longitudinal myocytes (Figs. 21, 25). An additional cell type, vacuolar cells, has appeared at this stage (Figs. 21, 25). These cells, which are not numerous, are directly connected neither to the mesothelium nor to the muscle layer, but are scattered within the connective tissue outer area, occurring singly or in small clusters of two or three cells. Vacuolar cells are limited by a basal lamina. Their cytoplasm is packed with vacuoles about 0.6 μm in diameter that enclose an electron-lucent heterogeneous material (Fig. 21).

The connective tissue layer still contains numerous type 3 pseudopodial cells that are widely distributed from the mesothelium to the inner epithelium (Figs. 22–25). In its inner area, it encloses densely packed collagen fibers that start to be organized in helixes parallel to the long axis of the tubule. Intermingled with the collagen fibers are slender neurosecretory-like cell processes whose cytoplasm is filled



Figures 17–19. *Holothuria forskali.* Ultrastructure of stage 4 regenerating Cuvierian tubules. BL, basal lamina; CM, circular myocyte; G, Golgi apparatus; GC, granular cell; LM, longitudinal myocyte; MI, mitochondrion; N, nucleus; NP, nerve plexus; PC, peritoneocyte; RER, rough endoplasmic reticulum; SG, secretory granule; SL, secondary lysosome. **Figure 17.** Longitudinal section through the mesothelium (bar = 2 μm). **Figure 18.** Intraconnective type 3 pseudopodial cell (bar = 1 μm). **Figure 19.** Detailed view of the cytoplasm of a type 3 pseudopodial cell (the arrowhead indicates a spheroidal distention of a proximal Golgi cisterna; bar = 0.2 μm).

with electron-dense, membrane-bound granules (about 0.2 μm in diameter; Fig. 22). These processes are limited by a basal lamina.

Due to the considerable development of the connective tissue layer, the tubule lumen is narrow with a highly convoluted limiting epithelium. Each epithelial cell possesses one or two intraconnective, basal processes that enclose a few large spherules with a heterogeneous content (Figs. 23–25).

Study of cell proliferation

The distribution and abundance of proliferating cells at each regeneration stage were investigated by immunohistochemistry after BrdU incorporation, and by autoradiography after ^{3}HT incorporation. The intensity and pattern of cellular labeling were identical with both methods. The results show that, in the four first stages of regeneration, labeled nuclei are uniformly distributed in the three tissue layers of the regenerating tubule; *i.e.*, the mesothelium, the connective tissue layer, and the inner epithelium (Figs. 26–29). There is no evidence of a well-defined cell proliferation site. Labeling indices indicated that, in the three tissue layers, DNA-synthesizing cells are most abundant at the beginning of the regenerative process, their number then decreasing regularly in the successive regeneration stages (Fig. 26). In the connective tissue layer, however, the labeling index stays maximum until stage 3 and then regularly decreases (Fig. 26). At stage 5, very few labeled cells are observed in the three tissue layers, although the regenerating tubule measures only half of its final size. Labeled cells were never observed in a non-regenerating Cuvierian tubule.

Proliferating cells were identified by observation of consecutive semithin and ultrathin sections, the former being treated by autoradiography after ^{3}HT incorporation. In the

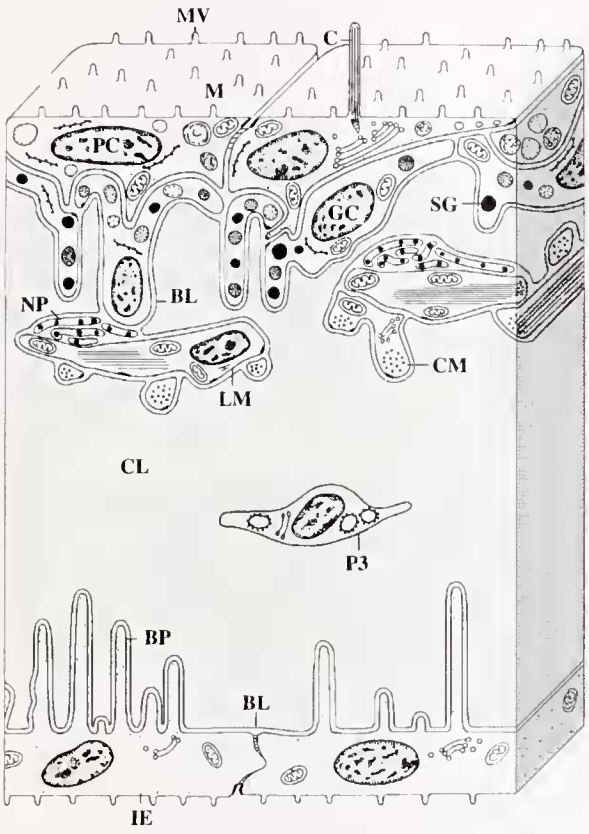


Figure 20. *Holothuria forskali.* Reconstruction of a longitudinal section through the wall of a stage 4 regenerating Cuvierian tubule (not to scale). BL, basal lamina; BP, epithelial cell basal process; C, cilium; CL, connective tissue layer; CM, circular myocyte; IE, internal epithelium; GC, granular cell; LM, longitudinal myocyte; M, mesothelium; MV, microvillus; NP, nerve plexus; P3, type 3 pseudopodial cell; PC, peritoneocyte; SG, secretory granule.

mesothelium, proliferating cells are present from regeneration stage 1 to stage 4. In all these stages, labeled nuclei were always observed in undifferentiated cells and peritoneocytes (Figs. 30, 31). No labeling was detected in myocytes and granular cells, whatever the regeneration stage considered. Labeled cells were observed in the connective tissue layer until the regenerating tubule becomes a small functional tubule (*i.e.*, end of stage 5). These cells were always pseudopodial cells (Figs. 30, 31) and never spherulocytes. Among the three types of pseudopodial cells, labeled nuclei were observed principally, but not exclusively, in type 2 cells (Figs. 29–31). As for the inner epithelium, the labeling was always detected in epithelial cells that had not yet developed piles of spherules in their intraconnective basal processes.

Discussion

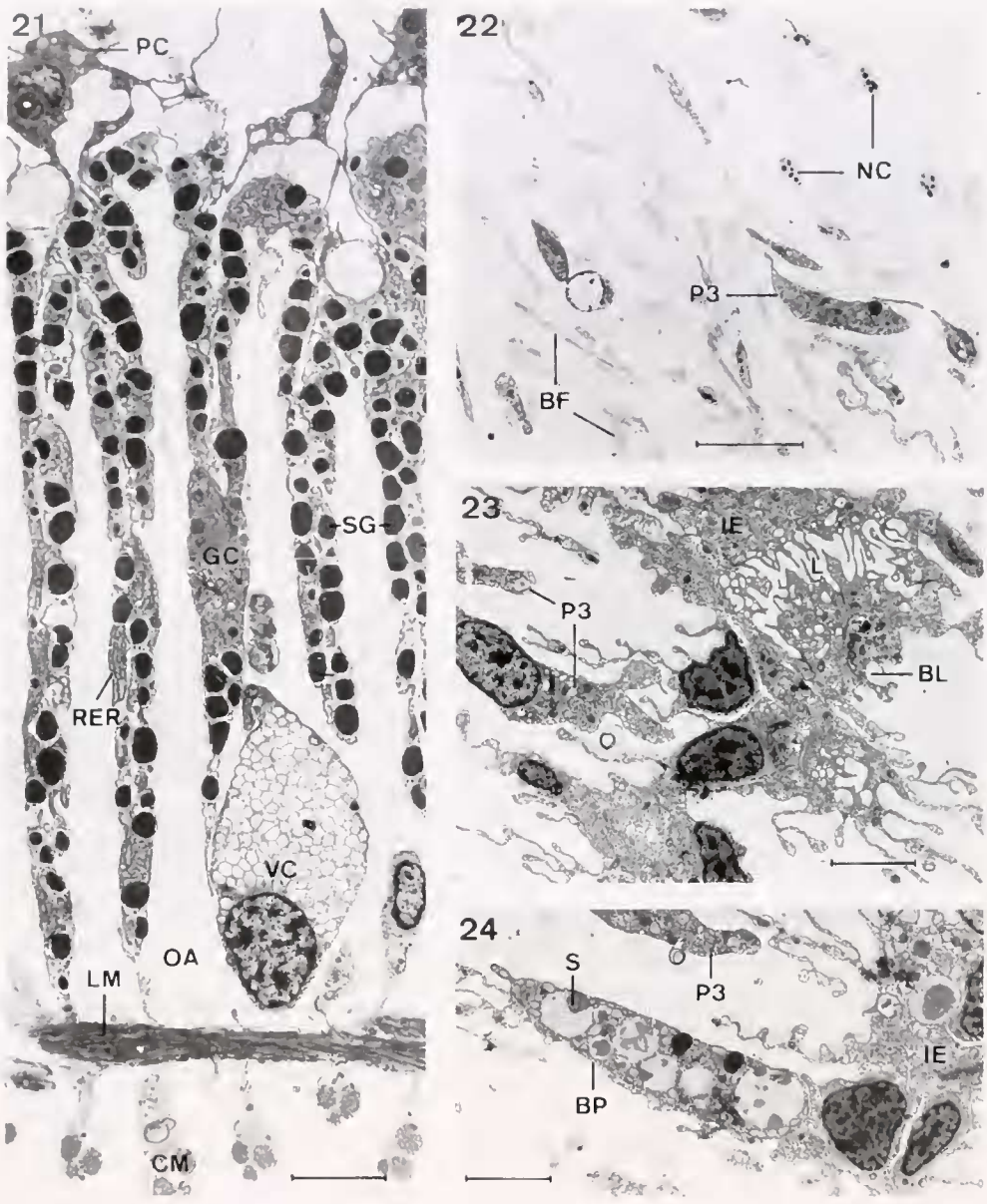
Echinoderms in general, and holothuroids in particular, exhibit a remarkable ability to regenerate a missing part of the body (Hyman, 1955). Regeneration in echinoderms generally occurs after evisceration, autotomy, or fission (Emson and Wilkie, 1980). In sea cucumbers, the best studied regenerative model is the intestine regeneration after evisceration (Dawbin, 1949; Mosher, 1956; Bai, 1971; Tracey, 1972; Garcia-Arraras *et al.*, 1998). In this model, formation of the new gut may be subdivided into three successive phases: initial repair, true regeneration, and growth. The same phases were observed in the replacement process of autotomized Cuvierian tubules in *Holothuria forskali.*

Phases of Cuvierian tubule regeneration

The repair phase, which includes the overall 48-h post-autotomy period, comprises wound closure and histolysis of the damaged tissues. During this phase, no clotting of coelomocytes was observed, contrary to what usually occurs during repair of injured tissues in echinoderms (Smith, 1981). This lack of coelomocyte plugging may reflect the rapid closure of the relatively limited wound by the contraction of the circular muscles of the tubule peduncle. Wound closure is followed by re-epithelialization on both the coelomic and luminal sides of the basal part of the left respiratory tree. Phagocytosis of the damaged tissues then takes place within the connective tissue layer. This is the role of the many large and active phagocytic cells observed during this phase. Such cells have been described at wound sites in the five echinoderm classes (Gibson and Burke, 1983; Mladenov *et al.*, 1989; Candia Carnevali *et al.*, 1993; Dubois and Ghyoot, 1995). These cells are generally considered to be derived from coelomic amoebocytes that migrate to the wound site (Smith, 1971a; Dubois and Ghyoot, 1995).

The regenerative process *sensu stricto* starts right after wound healing and takes 3 to 4 weeks to complete. Five stages of regeneration may be recognized. Stage 1 can be defined by the appearance of a thickening of the mesothelium at the site where autotomy took place. This thickening will be the starting point of the new tubule formation. Stage 2 corresponds to the initial appearance of a lumen within the regenerating tubule. The tubule wall therefore acquires its characteristic trilayered structure, which consists of an outer mesothelium, a connective tissue layer, and an inner epithelium. Stage 3 involves a tissue layer proportioning in which the connective tissue layer grows to become the thickest of the three tissue layers, as in non-regenerating Cuvierian tubules. Stage 4 is characterized by the acquisition of the typical tubule shape. Finally, stage 5 is reached when the regenerating tubule becomes functional.

Regeneration is followed by a growth phase. Indeed, the functional tubule formed at the end of the last regeneration stage is only half the size of a non-regenerating Cuvierian tubule. It thus continues to grow for about one more week until it reaches its final size.



Figures 21–24. *Holothuria forskali.* Ultrastructure of stage 5 regenerating Cuvierian tubules. BF, bundle of collagen; BL, basal lamina; BP, epithelial cell basal process; CM, circular myocyte; IE, internal epithelium; GC, granular cell; L, lumen; LM, longitudinal myocyte; NC, neurosecretory-like cell; OA, outer area of the connective tissue layer; P3, type 3 pseudopodial cell; PC, peritoneocyte; RER, rough endoplasmic reticulum; S, spherule; SG, secretory granule; VC, vacuolar cell. **Figure 21.** Longitudinal section through the mesothelium and the muscle layer (bar = 3 μm). **Figure 22.** Longitudinal section through the inner area of the connective tissue layer (bar = 4 μm). **Figure 23.** Longitudinal section through the inner epithelium (bar = 3 μm). **Figure 24.** Detailed view of an intraconnective basal process of a cell of the inner epithelium (bar = 3 μm).

Cell cycle activity and origin of tissue layers

The use of BrdU[5] and ^{3}HT incorporation has provided further details concerning the role of cell proliferation in Cuvierian tubule regeneration and also, indirectly, concerning the possible contributions of migration and differentiation during regeneration.

In *H. forskali*, our results showed that cell proliferation occurs within each of the three constitutive tissue layers of the regenerating tubule and during the whole regenerative phase, although it was observed only in the mesothelium at stage 1 and only in the connective tissue layer at stage 5. Conversely, no labeled nucleus was observed after stage 5

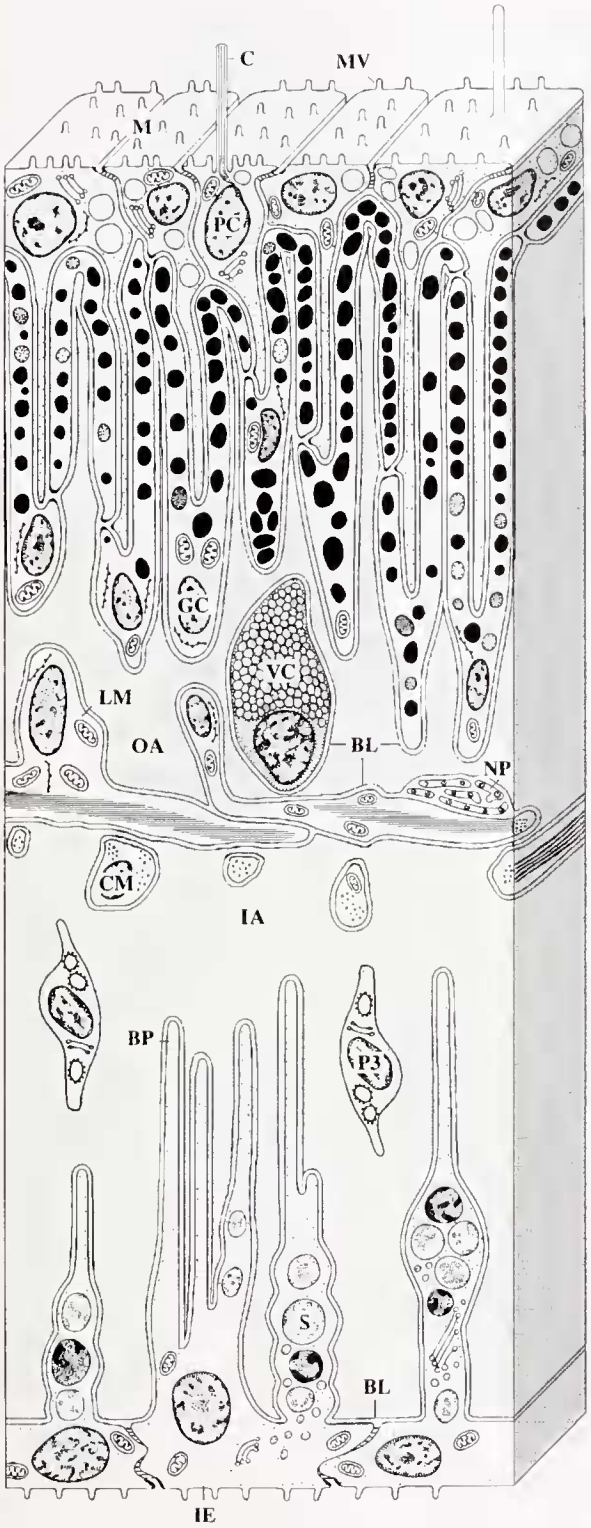


Figure 25. *Holothuria forskali.* Reconstruction of a longitudinal section through the wall of a stage 5 regenerating Cuvierian tubule (not to scale). BL, basal lamina; BP, epithelial cell basal process; C, cilium; CM, circular myocyte; IA, inner area of the connective tissue layer; IE, internal epithelium; GC, granular cell; LM, longitudinal myocyte; M, mesothelium; MV, microvillus; NP, nerve plexus; OA, outer area of the connective tissue layer; P3, type 3 pseudopodial cell; PC, peritoneocyte; S, spherule; VC, vacuolar cell.

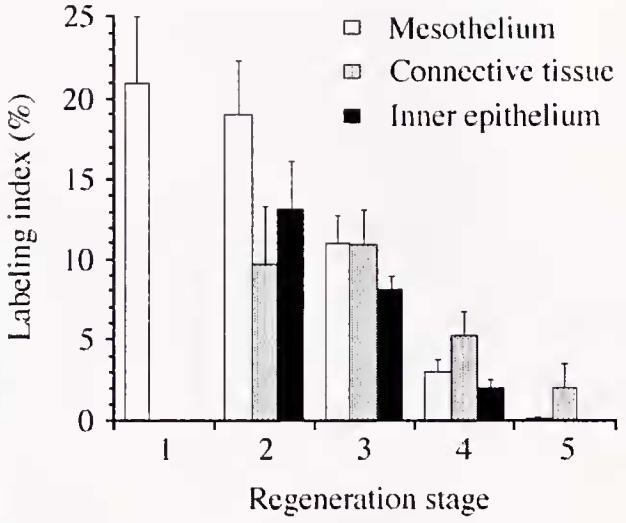
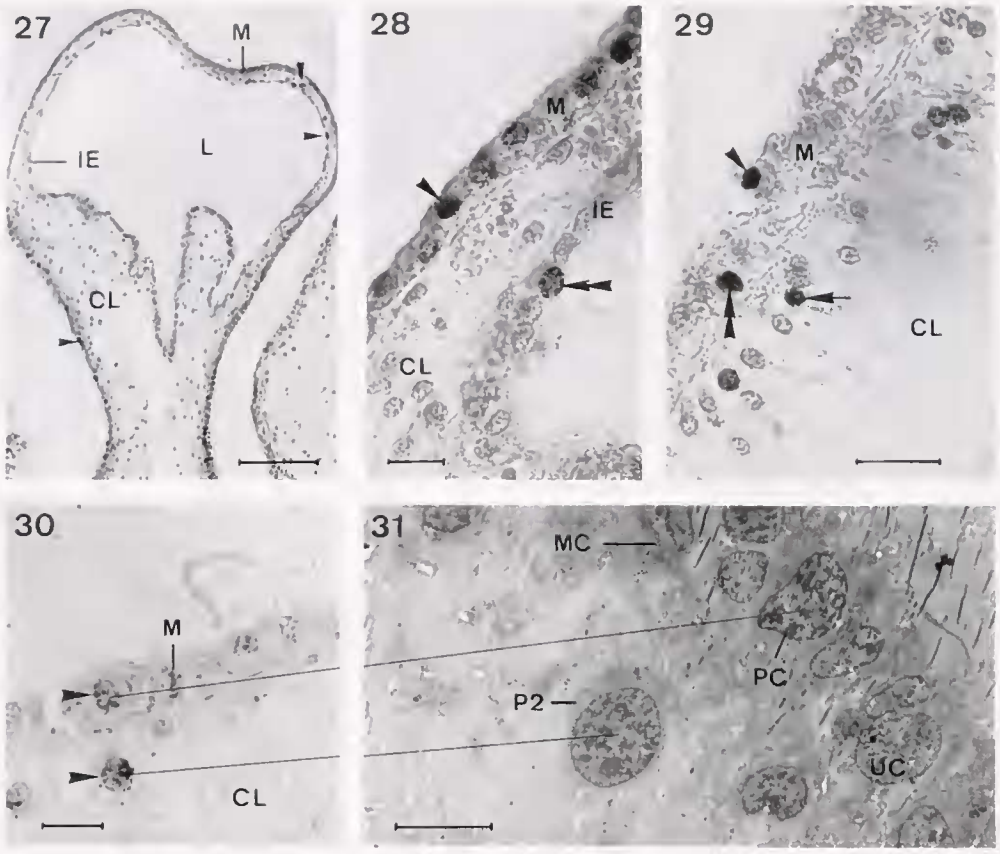


Figure 26. Labeling indices (mean ± SD, $n = 4$) for proliferating cells in the different tissue layers at each stage of Cuvierian tubule regeneration (BrdU/anti-BrdU method).

(*i.e.*, when the regenerating tubule becomes functional), indicating that the growth phase proceeds only by an increase of cell volume, without cell division. Moreover, the absence of DNA-synthesizing cells in non-regenerating Cuvierian tubules indicates that there is no cellular "turnover" and that the cell populations which constitute the tubule tissues must be considered as static (Messier and Leblond, 1960).

During regeneration, the mesothelium is the tissue layer in which cell proliferation is the most precocious and the most important, involving both undifferentiated cells and peritoneocytes. At stage 1, there is an accumulation of actively proliferating undifferentiated cells that form the mesothelial thickening characteristic of this stage. As regeneration proceeds, the percentage of labeled nuclei in the mesothelium regularly decreases in parallel with the differentiation of granular cells on the one hand and myocytes on the other hand. Nucleus labeling was never observed in these two cell types, which therefore do not divide. We were not able to determine the origin of undifferentiated cells, but they probably originate from peritoneocytes. These cells would thus contribute significantly to the regenerative process through dedifferentiation, proliferation, and redifferentiation into other cell types (in this case, granular cells and myocytes). Their relative totipotence in echinoderms is manifested by their ability to proliferate actively during regeneration (Candia Carnevali *et al.*, 1997; present study) and to transdifferentiate into free coelomocytes or myocytes (Vanden Bossche and Jangoux, 1976; Dolmatov *et al.*, 1996; respectively).

In their description of the ultrastructure of non-regenerating Cuvierian tubules, VandenSpiegel and Jangoux (1987) suggested that the intraconnective myocytes were derived



Figures 27–31. *Holothuria forskali.* Detection of proliferating cells in the regenerating Cuvierian tubules. CL, connective tissue layer; IE, internal epithelium; L, lumen; M, mesothelium; MC, myocyte; P2, type 2 pseudopodial cell; PC, peritoneocyte; UC, undifferentiated cell. **Figure 27.** Longitudinal section through a stage 2 regenerating tubule (BrdU/anti-BrdU method) showing labeled nuclei in the three tissue layers (arrowheads; bar = 50 μm). **Figure 28.** Detailed view of the wall of a stage 2 regenerating tubule (BrdU/anti-BrdU method) showing labeled nuclei in the mesothelium (single arrowhead) and in the inner epithelium (double arrowhead) (bar = 5 μm). **Figure 29.** Section through the wall of a stage 3 regenerating tubule (BrdU/anti-BrdU method) showing labeled nuclei in the mesothelium (peritoneocyte, single arrowhead; and undifferentiated cell, double arrowhead) and in the connective tissue layer (type 2 pseudopodial cell, arrow) (bar = 8 μm). **Figures 30 and 31.** Consecutive semithin and ultrathin sections through a stage 3 regenerating tubule. ^{3}HT incorporation in the tubule tissues was revealed by autoradiography on the semithin section (arrowheads, Fig. 29; bar = 5 μm) and the DNA-synthesizing cells were unambiguously identified by observation of the ultrathin section in TEM (Fig. 30; bar = 4 μm).

from the mesothelium. However, they had no direct evidence to corroborate their hypothesis. Our study of regenerating tubules clearly indicates the mesothelial origin of the myocytes which, during the regenerative process, first differentiate as myoepithelial cells and then migrate in the connective tissue layer. This is consistent with the hypothesis of Rieger and Lombardi (1987) on the mesothelial origin of nonepithelial myocytes in echinoderms. This speculative hypothesis was criticized by Cavey and Wood (1990), but has since been strengthened by the work of Stauber (1993) on echinoid lantern muscles, that of Dolmatov *et al.* (1996) on holothuroid longitudinal muscle bands, and now by ours on holothuroid Cuvierian tubule muscles.

The nerve plexus appears early in regeneration and consists only of nerve processes; no cell body was observed. This plexus is first basimesothelial and then migrates in the connective tissue layer with the myocytes. This explains why, in non-regenerating tubules, the nerve plexus is never observed within the mesothelium. Vacuolar cells were originally described as egg-shaped structures and interpreted as sections through a spiral nerve (VandenSpiegel and Jangoux, 1987). However, our reexamination of these structures in the regenerating tubules suggests that they are clusters of vacuolar cells. Because these cells first appear close to the mesothelium and are surrounded by a basal lamina, their origin is probably in the mesothelium. Like myocytes and granular cells, they presumably differentiate from undifferentiated cells. These cells have also been ob-

served in the Cuvierian tubules of other holothuroid species (VandenSpiegel and Jangoux, 1988), but their function remains enigmatic.

In the connective tissue layer, spherulocytes and pseudopodial cells co-occur at the beginning of the regeneration. However, although the pseudopodial cells appear to proliferate actively, spherulocytes show no sign of DNA synthesis and disappear early in the regenerative process. Nevertheless, they presumably play an important role in the early stages of Cuvierian tubule restoration. Indeed, most studies of wound repair in holothuroids have revealed the importance of spherulocytes (Cowden, 1968; Menton and Eisen, 1973). The function generally ascribed to these cells is the formation and maintenance of the ground substance of the extracellular matrix (Fontaine and Lambert, 1977; Byrne, 1986; Jans *et al.*, 1996). In addition, spherulocytes have been put forward as producers of antibacterial compounds and chemotactic agents for other mesenchymatous cells (see Smith, 1981, for review). The latter could account for the recruitment of type 1 pseudopodial cells at the beginning of Cuvierian tubule regeneration.

Three types of pseudopodial cells follow one another in the tubule connective tissue during regeneration. Type 1 cells have all the characteristics of echinoderm phagocytes (*e.g.*, see Dubois and Ghyoot, 1995). The collagen-containing secondary lysosomes observed in some of these cells suggest they may have a fibroclastic function, cleaning up the connective tissue compartment before new collagen synthesis starts. Type 2 cells are characterized by a more rounded shape, a high nuclear:cytoplasmic ratio, and a conspicuous juxtanuclear centriole—all features suggesting that these cells are rather undifferentiated (Fontaine and Lambert, 1977; Smith, 1981). This notion is further supported by the active division of these cells. Type 3 cells possess distended RER cisternae and Golgi stacks ending in spheroidal distentions. RER with distended cisternae has been described both in proven and presumptive echinoderm fibroblasts (Dubois and Ghyoot, 1995; Heinzeller and Welsch, 1994; respectively). Golgi with spheroidal distentions, on the other hand, are a diagnostic feature of vertebrate fibroblasts (Weinstock and Leblond, 1974). We have not observed all the stages of Golgi distentions, including the final collagen granule. Nevertheless, the ultrastructural characteristics of type 3 pseudopodial cells, together with their presence in the regenerating connective tissue close to the time when the collagen fibers appear, strongly support a fibroblastic function for these cells. According to the sequence of appearance of the three types of pseudopodial cells, type 2 cells are probably dedifferentiated type 1 cells that then actively proliferate before differentiating into type 3 cells. Phagocytic cells, undifferentiated mesenchymatous cells, and fibroblasts would thus share lineage relationships, as is the case in echinoids (Dubois and Ghyoot, 1995). Once the regenerating tubule has reached its definitive size, the collagen synthesis presumably stops, as evidenced by the lack of mesenchymatous cells in non-regenerating Cuvierian tubules (VandenSpiegel and Jangoux, 1987). The origin of the tridimensional organization of the collagen in parallel helixes is not clear, but it could be the result of the particular association observed at regeneration stage 3 between type 2 pseudopodial cells and the mesothelium.

In the inner epithelium, cells also divide actively, but only when they have not yet differentiated spherules in their basal intraconnective processes ("undifferentiated epithelial cells"). It appears, therefore, that in the three tissue layers of the Cuvierian tubules, regeneration proceeds by dedifferentiation, then proliferation, and finally differentiation.

Cuvierian tubule regeneration: morphallaxis vs. *epimorphosis*

Regeneration has been described classically as proceeding by one or the other of two mechanisms: (i) morphallaxis, in which cells differentiate or migrate from existing populations, and (ii) epimorphosis, in which mitosis occurs and new cells are produced that either directly replace those lost or form a blastema that goes on to differentiate and replace lost tissue (Bonasoro *et al.*, 1998). In echinoderms, the relative importance of morphallaxis and epimorphosis seems to be variable. Regeneration of longitudinal muscle bands in holothuroids proceeds without cell proliferation and represents a case of cellular morphallaxis (Dolmatov *et al.*, 1996). Similarly, regeneration of the intestine after fission in the apodid holothuroid *Leptosynapta* is an entirely morphallactic mechanism; in this case, the remaining portion of the intestine is remodeled to form a functionally complete organ (Smith, 1971a, b; Gibson and Burke, 1983). Conversely, regeneration of the arm in the crinoid *Antedon mediterranea* is an epimorphic process in which a regenerative blastema is formed (Candia Carnevali *et al.*, 1993, 1995, 1997). Most regenerative processes in echinoderms, however, appear to involve a combination of morphallactic and epimorphic mechanisms. This is the case for the intestine regeneration after evisceration in aspidochirote and dendrochirote holothuroids (Dolmatov, 1992; Garcia Arraras *et al.*, 1998), as well as for the arm regeneration in asteroids (Mladenov *et al.*, 1989; Moss *et al.*, 1998).

Regeneration of the Cuvierian tubules in *H. forskali* also appears to involve both epimorphosis and morphallaxis. Initial regeneration events occur by epimorphosis, cell proliferation being essential to the regenerative process. The mesothelial thickening appearing at stage 1 could be compared to a blastema, that is, an accumulation of undifferentiated cells capable of proliferation and differentiation. However, it is not a true blastema, giving rise to all the tissue layers in the new tubule; rather it is a transitory blastema-like structure that contributes only to the regeneration of the mesothelium, forming mesothelial cells (peri-

toneocytes and granular cells) and mesothelium-derived cells (myocytes and vacuolar cells). This structure does not contribute to the regeneration of the other two tubule layers—*i.e.*, the connective tissue layer and the inner epithelium—that each enclose their own population of dividing cells. As regeneration proceeds, the percentage of dividing cells decreases, and in the late stages, tubule histogenesis occurs mainly by morphallaxis, with newly differentiated cells migrating from the mesothelium into the connective tissue layer.

The dynamics of Cuvierian tubule regeneration and its implication for the defense mechanism

In holothuroid species that contain Cuvierian tubules, the structures are generally present in large numbers. For example, individuals of *H. forskali* may have between 200 and 600 tubules, depending on their size (VandenSpiegel and Jangoux, 1987). When gently stimulated they expel only a few tubules, but if the stimulation is stronger, tubules can be discharged several times in succession (Bakus, 1968; present study). However, in both cases, only a fraction of the total tubule number will be used. Such a behavior allows a sparing use of the defensive organ (VandenSpiegel and Jangoux, 1987). After expulsion and autotomy, Cuvierian tubules are readily regenerated. In *H. forskali*, our study showed that the complete regeneration of the autotomized tubules, namely the formation of functional, fully developed tubules, takes about 5 weeks. This period starts with the actual beginning of the regenerative process and not with the expulsion and autotomy of the tubules. Indeed, after a gentle stimulation, when only a few (about 15) tubules have been expelled, there is often a latent period that may last up to 3 weeks before the regeneration starts. In contrast, after a strong stimulation, when many (up to 300) tubules have been expelled, the regenerative process starts immediately, but proceeds by successive waves of 10 to 30 tubules that begin to regenerate at 10-day intervals. This pattern of regeneration is advantageous for the sea cucumber because it allows a staggered spending of the energy necessary for regeneration. Holothuroid Cuvierian tubules thus constitute a very efficient defensive mechanism. Indeed, in addition to their remarkable structural organization, which accounts for their adhesive and mechanical properties (VandenSpiegel and Jangoux, 1987), their large number, sparing use, and particular regeneration dynamics also make them an almost inexhaustible line of defense maintained at limited energy cost.

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Literature Cited

Bai, M. M. 1971. Regeneration in the holothurian, *Holothuria scabra* Jager. *Indian J. Exp. Biol.* **9:** 467–471.

Bakus, G. J. 1968. Defensive mechanisms and ecology of some tropical holothurians. *Mar. Biol.* **2:** 23–32.

Bonasoro, F., M. D. Candia Carnevali, C. Moss, and M. C. Thorndyke. 1998. Epimorphic *versus* morphallactic mechanisms in arm regeneration of crinoids and asteroids: pattern of cell proliferation and cell lineage. Pp. 13–18 in *Echinoderms: San Francisco*, R. Mooi and M. Telford, eds. Balkema, Rotterdam.

Byrne, M. 1986. The ultrastructure of the morula cells of *Eupentacta quinquesemita* (Echinodermata, Holothuroidea) and their role in the maintenance of the extracellular matrix. *J. Morphol.* **188:** 179–189.

Candia Carnevali, M. D., E. Lucca, and F. Bonasoro. 1993. Mechanisms of arm regeneration in the feather star *Antedon mediterranea:* healing of wound and early stages of development. *J. Exp. Zool.* **267:** 299–317.

Candia Carnevali, M. D., F. Bonasoro, E. Lucca, and M. C. Thorndyke. 1995. Pattern of cell proliferation in the early stages of arm regeneration in the feather star *Antedon mediterranea*. *J. Exp. Zool.* **272:** 464–474.

Candia Carnevali, M. D., F. Bonasoro, and A. Biale. 1997. Pattern of bromodeoxyuridine incorporation in the advanced stages of arm regeneration in the feather star *Antedon mediterranea*. *Cell Tissue Res.* **289:** 363–374.

Cavey, M. J., and R. L. Wood. 1990. Organization of the adluminal and retractor cells in the coelomic lining from the tube foot of a phanerozonian starfish, *Luidia foliolata*. *Can. J. Zool.* **69:** 911–923.

Cowden, R. R. 1968. Cytological and histochemical observations on the connective tissue cells and cutaneous wound healing in the sea cucumber *Stichopus badionotus*. *J. Invertebr. Pathol.* **10:** 151–159.

Dawbin, W. H. 1949. Auto-evisceration and regeneration of viscera in holothurian *Stichopus mollis* (Hutton). *Trans. R. Soc. N.Z.* **77:** 497–523.

Dolmatov, I. Y. 1992. Regeneration of aquapharyngial complex in the holothurian *Eupentacta fraudatrix* (Holothuroidea, Dendrochirota). *Monogr. Dev. Biol.* **23:** 40–50.

Dolmatov, I. Y., M. G. Eliseikina, A. A. Bulgakov, T. T. Ginanova, N. E. Lamash, and V. P. Korchagin. 1996. Muscle regeneration in the holothurian *Stichopus japonicus*. *Roux's Arch. Dev. Biol.* **205:** 486–493.

Dubois, P., and M. Ghyoot. 1995. Integumentary resorption and collagen synthesis during regression of headless pedicellariae in *Sphaerechinus granularis* (Echinodermata, Echinoidea). *Cell Tissue Res.* **282:** 297–309.

Emson, R. H., and I. C. Wilkie. 1980. Fission and autotomy in echinoderms. *Oceanogr. Mar. Biol. Annu. Rev.* **18:** 155–250.

Flammang, P. 1996. Adhesion in echinoderms. Pp. 1–60 in *Echinoderm Studies*, Vol. 5, M. Jangoux and J. M. Lawrence, eds. Balkema, Rotterdam.

Fontaine, A. R., and P. Lambert. 1977. The fine structure of leucocytes of the holothurian *Cucumaria miniata*. *Can. J. Zool.* **55:** 1530–1544.

Gabe, M. 1968. *Techniques histologiques*. Masson, Paris.

Garcia-Arraras, J. E., L. Estrada-Rodgers, R. Santiago, I. I. Torres, L. Diaz-Miranda, and I. Torres-Avillan. 1998. Cellular mechanisms of intestine regeneration in the sea cucumber, *Holothuria glaberrima* Selenka (Holothuroidea: Echinodermata). *J. Exp. Zool.* **281:** 288–304.

Gibson, A. W., and R. D. Burke. 1983. Gut regeneration by morphal-

laxis in the sea cucumber *Leptosynapta clarki* (Heding, 1928). *Can. J. Zool.* **61:** 2720–2732.

Hacker, G., D. Springall, S. VanNoorden, A. Bishop, L. Grimelius, and J. Polak. 1985. The immunogold-silver staining method—a powerful tool in histopathology. *Virchows Arch. B* **406:** 449–461.

Heinzeller, T., and U. Welsch. 1994. Crinoidea. Pp. 9–148 in *Microscopic Anatomy of Invertebrates, Vol. 14: Echinodermata,* F. W. Harrison and F.-S. Chia, eds. Wiley-Liss, New York.

Hill, R. B., and D. Reinschmidt. 1976. Relative importance of the antioxidant and anesthetic properties of propylene phenoxetol in its action as a "preservatie" for living holothurians. *J. Invertebr. Pathol.* **28:** 131–135.

Humphrey, C. D., and F. E. Pittman. 1974. A simple methylene blue-azur II-basic fushin stain for epoxy-embedded tissue secretion. *Stain Technol.* **49:** 9–14.

Hyman, L. H. 1955. *The Invertebrate, Vol. 4 Echinodermata.* McGraw-Hill, New York.

Jans, D., P. Dubois, and M. Jangoux. 1996. Defensive mechanisms of holothuroids (Echinodermata): formation, role, and fate of intracoelomic brown bodies in the sea cucumber *Holothuria tubulosa. Cell Tissue Res.* **283:** 99–106.

Laurent, G., G. Toubeau, J. A. Heuson-Stiennon, P. Tulkens, and P. Maldague. 1988. Kidney tissue repair after nephrotoxic injury: biochemical and morphological characterization. *Crit. Rev. Toxicol.* **19:** 147–183.

Menton, D. N., and A. Z. Eisen. 1973. Cutaneous wound healing in the sea cucumber, *Thyone briareus. J. Morphol.* **141:** 185–204.

Messier, B., and C. P. Leblond. 1960. Cell proliferation and migration as revealed by autoradiography after injection of thymidine-^{3}H into male rats and mice. *Am. J. Anat.* **106:** 247–265.

Mladenov, P. V., B. Bisgrove, S. Asotra, and R. D. Burke. 1989. Mechanisms of arm tip regeneration in the sea star, *Leptasterias hexactis. Roux's Arch. Dev. Biol.* **198:** 19–28.

Mosher, C. 1956. Observations on evisceration and visceral regeneration in the sea-cucumber, *Actinopyga agassizi* Selenka. *Zoologica (N.Y.)* **41:** 17–26.

Moss, C., A. J. Hunter, and M. C. Thorndyke. 1998. Patterns of bromodeoxyuridine incorporation and neuropeptide immunoreactivity during arm regeneration in the starfish *Asterias rubens. Phil. Trans. R. Soc. Lond.* **353:** 421–436.

Müller, M. E. G., R. K. Zahn, and K. Schmid. 1970. Morphologie und Funktion der Cuvierian Organe von *Holothuria forskali* Delle Chiaje (Echinodermata: Holothuroidea: Aspidochirota: Holothuriidae). *Z. Wiss. Zool.* **181:** 219–231.

Rieger, R. M., and J. Lombardi. 1987. Ultrastructure of the coelomic lining in echinoderm podia: significance for concepts in the evolution of muscle and peritoneal cells. *Zoomorphology* **107:** 191–208.

Smiley, S. 1994. Holothuroidea. Pp. 401–471 in *Microscopic Anatomy of Invertebrates, Vol. 14: Echinodermata,* F. W. Harrison and F.-S. Chia, eds. Wiley-Liss, New York.

Smith, G. N. 1971a. Regeneration in the sea cucumber *Letosynapta.* I. The process of regeneration. *J. Exp. Zool.* **177:** 319–330.

Smith, G. N. 1971b. Regeneration in the sea cucumber *Letosynapta.* II. The regenerative capacity. *J. Exp. Zool.* **177:** 331–342.

Smith, V. J. 1981. The echinoderms. Pp. 514–562 in *Invertebrate Blood Cells,* N. A. Ratcliffe and A. F. Rowley, eds. Academic Press, New York.

Stauber, M. 1993. The lantern of Aristotle: organization of its coelom and origin of its muscles (Echinodermata, Echinoidea). *Zoomorphology* **113:** 137–151.

Tracey, D. J. 1972. Evisceration and regeneration in *Thyone okeni* (Bell, 1884). *Proc. Linn. Soc. N. S. W.* **97:** 72–81.

Vanden Bossche, M., and M. Jangoux. 1976. Epithelial origin of starfish coelomocytes. *Nature* **261:** 227–228.

VandenSpiegel, D., and M. Jangoux. 1987. Cuvierian tubules of the holothuroid *Holothuria forskali* (Echinodermata): a morphofunctional study. *Mar. Biol.* **96:** 263–275.

VandenSpiegel, D., and M. Jangoux. 1988. Les tubes de Cuvier d'*Holothuria mammata* Grube, 1840 (Holothuroidea, Echinodermata). *Ann. Soc. R. Zool. Belg.* **118:** 191–198.

VandenSpiegel, D., D. Nonclercq, and G. Toubeau. 1991. Immunocytochemical detection of bromodeoxyuridine in proliferating cells of regenerating Cuvierian tubules of *Holothuria forskali.* Pp 81–85 in *Echinoderm Research 1991,* L. Scalera-Liaci and C. Canicatti, eds. Balkema, Rotterdam.

Weinstock, M., and C. P. Leblond. 1974. Synthesis, migration, and release of precursor collagen by odotonblasts as visualized by autoradiography after [^{3}H]proline administration. *J. Cell Biol.* **60:** 92–127.